Assessing population structure and gene flow in Montana wolverines (Gulo gulo) using assignment-based approaches

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Abstract
In North America, wolverines once occupied a continuous range from Alaska southward to New Mexico. In the lower 48 states, small remnant populations remain only in the northwestern United States. Among these remnant populations, the Montana population has the highest probability of long-term persistence given its size and proximity to healthy populations in Canada. In this study, we evaluate population genetic structure and gene flow among Montana wolverines using 10 polymorphic microsatellite loci. Bayesian and frequency-based assignment tests revealed significant population substructure and provide support for at least three subpopulations in Montana. $F_{ST}$ values between subpopulations ranged from 0.08 to 0.10 and provide evidence for male-biased dispersal. The high degree of population substructure and low levels of gene flow contrast results from wolverine population genetic studies in less fragmented landscapes of Alaska and Canada. This study provides additional support for the hypothesis that large carnivore populations of Montana are becoming increasingly fragmented due to human development and disturbance.

Keywords: assignment test, gene flow, genetic structure Gulo gulo, wolverine

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Introduction
Accurately defining the spatial extent of a population is essential for determining the appropriate scale for conservation and management. Population boundaries are often defined a priori by topographic features such as rivers or mountains, or alternatively by political jurisdictions such as state and country borders. Wide-ranging species with continuous distributions often do not have clear population boundaries. This has been demonstrated in the genetic analyses of highly mobile carnivores including coyote (Canis latrans; Lehman & Wayne 1991), grey wolf (Canis lupus; Roy et al. 1994), jaguar (Panthera onca; Eizirik et al. 2001), cougar (Puma concolor; Sinclair et al. 2001), black bear (Ursus americanus; Paetkau & Strobeck 1994), brown bear (Ursus arctos; Paetkau et al. 1998) and wolverine (Gulo gulo; Kyle & Strobeck 2001). In these instances, using topographic or political boundaries to define populations may not be biologically meaningful given the great dispersal capabilities of carnivores.

Wolverines are medium-sized carnivores found in the mountains and open plains of tundra, taiga and forest zones (Wilson 1982; Nowak 1991). They are highly mobile with the ability to disperse up to 300 km within a year (Magoun 1985; Gardner 1985). In North America, wolverines have a fairly continuous northern distribution from Alaska to Hudson Bay, Canada with increasing fragmentation of occupied habitat in southern Canada (Kyle & Strobeck 2001). Populations in the conterminous United States are distributed patchily with remnant populations in Colorado, California, Idaho, Oregon, Washington, Wyoming and Montana (Hash 1987; Banci 1994). Increasing concern over the status of the populations in the lower 48 states prompted a petition to list the wolverine as threatened under the Endangered Species Act in the summer of 2000, but the decision has been postponed due to a lack of federal funding and staff (USFWS http://www.r6.fws.gov). The population in Montana is considered to be the largest and most stable population of wolverines, given its close proximity to healthy populations in Canada. This population is distributed across multiple mountain ranges, and population size and connectivity are unknown (Banci 1994).

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Genetic analysis of highly variable microsatellite loci can provide critical information about population structure and gene flow when coupled with new assignment test approaches (Paetkau et al. 1995; Pritchard et al. 2000). The assignment test was developed originally by Paetkau et al. (1995) to evaluate the degree of differentiation among populations. This test is based on frequency statistics that use the observed allele frequencies for each of the predefined reference populations to calculate the likelihood of each genotype in each of the populations. Individuals are then assigned to the population with the highest likelihood. This test has been used widely to estimate levels of genetic differentiation and gene flow among populations (Paetkau et al. 1995; Paetkau et al. 1998; Kyle et al. 2000).

Recent refinements of the assignment test have enabled researchers to expand its exploratory power. Favre et al. (1997) adapted the assignment test approach to evaluate sex biases in dispersal. Rannala & Mountain (1997) developed a Bayesian model to estimate the allele frequencies of each of the predefined reference populations. Cornuet et al. (1999) refined the assignment-based approach to exclude populations as sources if the reference population has not been sampled. In addition, Bayesian (Pritchard et al. 2000) and frequency-based (Vázquez-Dominguez et al. 2001) assignment tests have been developed that do not require predefined populations. These methods provide new approaches for defining population structure in species, such as wolverines, that have high dispersal abilities and populations with no detectable geographical or phenotypic distinctiveness.

To evaluate wolverine population structure and gene flow in Western Montana, data from 10 microsatellite loci were collected from 89 individuals, and these data were analysed using four different assignment test approaches. The objectives of our project were to (1) evaluate population substructure and delineate population boundaries for Montana wolverines; (2) assess levels of gene flow among the identified populations; and (3) examine evidence for sex biases in dispersal. Our results are used to develop recommendations for conservation and management of wolverines and to provide researchers with guidelines for using assignment test approaches to define populations and detect migrants.

Materials and methods

Sampling and DNA extraction

Tissue samples of harvested wolverines were collected by the Montana Department of Fish, Wildlife and Parks Research Laboratory. Eighty-nine wolverine tissue samples (43 females, 46 males) were collected from 1989 to 2000 and stored frozen until DNA extraction (Fig. 1a,b). Although samples were collected across a time span of 11 years (approx. two generations), there is no evidence to suggest that large demographic changes have occurred during this sampling period, which would alter allele frequencies; therefore, samples were combined across years. Sampling

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location was provided by trappers and converted to latitude and longitude for mapping purposes. Sex and age were recorded for each individual.

DNA was extracted from a 1-mm piece of tissue using the Qiagen tissue protocol (Qiagen Co., USA) with one modification. Buffer ATL was substituted with 1× nucleic acid purified lysis buffer (Applied Biosystems, USA), which aids in the lysis of older tissue samples (Guglich et al. 1994).

Microsatellite analysis

Ten microsatellite loci were amplified, Lut604 (Dallas & Piertney 1998), Ggu101B, Ggu216, Ggu234, Gg3, Gg4, Gg7, Gg14 (Davis & Strobeck 1998; Duffy et al. 1998), Ma-3 and Tt-4 (Davis & Strobeck 1998), using fluorescantly labelled primers. Relative concentrations of primers were adjusted by trial and error to create multiplexes. Three multiplex polymerase chain reactions (PCR) and one single PCR were performed. Multiplex 1 consisted of primers Ggu216 (0.5 µm) and Ggu234 (0.5 µm). A 15-µL PCR reaction was performed using 1.5 µL of genomic DNA, 2.5 mM of each dNTP, 1× Redtaq buffer (Sigma), 1.5 units of Redtaq polymerase (Sigma). Cycling was performed using a PTC-100 (MJ Research) with the following profile: 95 °C for 30 s, 40 cycles of 30 s at 90 °C, 30 s at 55 °C and 40 s at 72 °C, and a final 2-min extension at 72 °C. Multiplex 2 consisted of primers Ma-3 (0.17 µm), Gg14 (0.17 µm) and Tt4 (0.07 µm). PCR reaction conditions and profiles are identical to multiplex 1 except for a 60 °C annealing temperature.

Multiplex 3 consisted of primers Lut604 (0.23 µm), Gg7 (0.07 µm), Gg4 (0.53 µm) and Gg3 (0.17 µm). PCR reaction conditions and profiles are the same as multiplex 1 except for a 53 °C annealing temperature. Ggu101B was amplified alone with the following reaction conditions: 1.5 µL of genomic DNA, 0.67 µm primer, 2.5 mm of MgCl₂, 1.5 units of AmpliTaq Gold polymerase (Perkin-Elmer Cetus), 1× Cetus buffer, 2.5 mM of each dNTP in a 15-µL cocktail. The following PCR profile was used: 95 °C for 1 min, 45 cycles of 30 s at 90 °C, 30 s at 55 °C and 40 s at 72 °C and a final 2-min extension at 72 °C. All 10 loci were loaded into a single lane of 6% Long Ranger acrylamide gel and fragments were separated using an ABI 377 fluorescent detection system (Applied Biosystems). Bands were scored with the programs GENESCAN 3.0 and GENOTYPER 2.5 (Applied Biosystems).

Bayesian analysis using structure

The program STRUCTURE (version 2) was used to cluster individuals into respective subpopulations and reveal patterns of gene flow across the landscape (Pritchard et al. 2000, http://pritch.bsd.uchicago.edu). The first step in the analysis involves estimating the number of subpopulations (K). Five independent runs of K = 1–8 were performed at 200 000 MCMC repetitions and 200 000 burn-in period using no prior information and assuming correlated allele frequencies and admixture. The posterior probability was then calculated for each value of K using the estimated log-likelihood of K to choose the optimal K.

The second step of the analysis involved assigning individuals to each of the K subpopulations. Samples were placed into the respective subpopulation based upon the highest percentage of membership (q). When comparing results to other assignment test approaches, a threshold value of q ≥ 0.90 was chosen. This value provides a statistical cut-off within the range of suggested values in the literature (Manel et al. 2002) and indicates that ≥ 90% of ancestry can be attributed to the respective subpopulation. The assigned individuals were plotted on a map of Montana using ARCVIEW 3.2 (Esri Inc.) to examine geographical congruence. A 95% minimum convex polygon was used to cluster subpopulations using the Animal Movement extension in ARCVIEW 3.2 (Hooge & Eichenlauh 1997). Males and females also were tested independently using the same parameters described above to detect differences in structure. Each identified subpopulation was tested for Hardy–Weinberg equilibrium and linkage equilibrium using GENEPOP on the Web (Raymond & Rousset 1995). A sequential Bonferroni correction was used to adjust significance levels across multiple tests (Rice 1989).

Iterative assignment test

An iterative version of a frequency assignment test was used to assign individuals to subpopulations using a predefined number of subpopulations (Vázquez-Domínguez et al. 2001). Assignments were calculated using the frequency-based program of Paetkau et al. (1995) available at www.biology.uberta.ca/~jbrzusto/Doh.php. Three initial groups of 30, 30 and 29 individuals were constructed randomly based upon the optimal value of K = 3 determined in analyses using STRUCTURE. An iterative process of assignment was performed twice, using different starting groups, in which each round of assignment involved placing the ‘misassigned’ individuals into the assigned subpopulation based on the likelihood scores. This process was repeated iteratively until all samples were assigned to their respective subpopulations and the group composition was stable. Hardy–Weinberg equilibrium was evaluated for each iteration and grouping using GENEPOP on the Web (Raymond & Rousset 1995) with a sequential Bonferroni correction (Rice 1989). Genotype likelihood ratios (LOD scores) were calculated to evaluate statistical support for migrant classifications (Roques et al. 1997; Banks & Eichert 2000). This statistic calculates the logarithm (base 10) of the largest probability over the second largest probability. A LOD value > 1.0 indicates that the assigned population is 10 times more likely if LOD > 2.0, the assigned population
is 100 times more likely and if LOD > 3.0, the assigned population is 1000 times more likely.

**Evaluation of migrants using GENECLASS**

Assignment of individuals using predefined subpopulations was performed by two methods using the program GENECLASS 1.0.02 (Cornuet et al. 1999; http://www.ensam.inra.fr/URLB). The results of STRUCTURE at three subpopulations were entered as the reference populations. The frequency model (Paetkau et al. 1995) and the Bayesian model (Rannala & Mountain 1997) were used in the analysis with 'Leave one out' option (Cornuet et al. 1999).

Genotype likelihood ratios were calculated as above to evaluate statistical support (value > 1.0) and a threshold value of \( P < 0.10 \) was chosen for population assignment. If the probability of assignment is lower than the chosen threshold, the individual cannot be definitely assigned to a specific population.

**Assignment indices (AI\(_c\))**

Assignment indices were generated for each individual to evaluate potential sex biases in dispersal (Favre et al. 1997) using the software available at www2.biology.ualberta.ca/jbrzusto/Doh.php (Paetkau et al. 1995). The three subpopulations defined by STRUCTURE were used in the analyses. Assignment index values were corrected for population differences by subtracting the population means after log transforming the data (AI\(_c\)). The mean AI\(_c\) was then calculated for males and females in each subpopulation.

**Indirect estimates of gene flow**

Indirect estimates of gene flow were calculated using Slatkin’s private allele method (Slatkin 1985) and Wright’s estimate of \( N_m \) (Wright 1943; \( N_m = \{1 - F_{ST}\}/4F_{ST}\)). Pairwise \( F_{ST} \) estimates were calculated for males and females in each of the three subpopulations using GENEPOP on the Web (Raymond & Rousset 1995). A randomization approach applied with FSTAT (Goudet et al. 2002) was used to test significance of both mean AIC and \( F_{ST} \) estimates for males and females in each of the subpopulations.

**Results**

**Detection of wolverine subpopulations in Montana**

To verify the validity of pooling samples collected across two generations, we evaluated the null hypothesis that allele frequency distributions were identical for samples collected in 1989–95 and 1996–2001 and no significant differences were detected. The Bayesian analysis using STRUCTURE clearly indicated the presence of substructure in this sample of wolverines (Table 1). The likelihood of the data was lowest at \( K = 1 \) (Ln = −1911.3). Two modes were observed, one at \( K = 3 \) (Ln = −1703.8) and one at \( K = 5 \) (Ln = −1688.2). At \( K = 3 \), each population had an average proportion of membership ranging from \( q = 0.89 \) to \( q = 0.91 \) with an asymmetric number of individuals assigned to each group (\( n = 18, 29, 43; \) Table 1). At \( K = 3 \), only one of the 30 tests for Hardy–Weinberg equilibrium was rejected at the \( \alpha = 0.05 \) level with a sequential Bonferroni correction and the null hypothesis of linkage equilibrium was not rejected for any of the 135 tests (\( P < 0.05 \)). At \( K = 5 \), a higher proportion of admixed individuals were observed with membership ranging from \( q = 0.45 \) to \( q = 0.85 \) (Table 1). Two of the 50 tests for Hardy–Weinberg equilibrium were rejected at the \( \alpha = 0.05 \) level with a sequential Bonferroni correction. Our results were plotted spatially for \( K = 3 \) and \( K = 5 \) to evaluate the geographical relationships of the samples in different genetic clusters (Fig. 1). Results for \( K = 3 \) show more cohesive and discreet clusters than for \( K = 5 \). Based on the analyses above and the recommendations of Pritchard & Wen 2002), \( K = 3 \) was chosen and the populations groupings in Fig. 1(a) were defined for Montana.

<table>
<thead>
<tr>
<th>K</th>
<th>Log P (k/x)</th>
<th>Variance log P (k/x)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−1911.3</td>
<td>19.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>−1794.2</td>
<td>71.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>−1703.8</td>
<td>99.1</td>
<td>0.905(29)</td>
<td>0.895(43)</td>
<td>0.919(18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>−1716.7</td>
<td>206.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>−1688.2</td>
<td>226.5</td>
<td>0.456(19)</td>
<td>0.454(18)</td>
<td>0.825(18)</td>
<td>0.646(24)</td>
<td>0.859(12)</td>
</tr>
<tr>
<td>6</td>
<td>−1688.6</td>
<td>602.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>−1726</td>
<td>351.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>−1779</td>
<td>471.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Population genetic substructure and membership was evaluated further by using an iterative version of the frequency-based assignment test (Vázquez-Domínguez et al. 2001) and setting the number of genetic groups at three. After 13 rounds of iterative assignment testing, group composition was stable and three groups of sizes 17, 35 and 37 were produced. The null hypothesis of Hardy–Weinberg equilibrium was rejected at four of the 30 tests \((\alpha = 0.05 \text{ with a sequential Bonferroni correction})\). Similar geographical groupings to STRUCTURE were evident, yet a higher degree of mixing was observed spatially (data not shown). 84\% of the sample groupings were concordant with the results from STRUCTURE \((K = 3)\). The 14 individuals that received conflicting population assignments are shown in Table 2. These individuals tend to have mixed ancestry, as demonstrated by comparing the average \(q\)-values for the 75 concordant placements \((q = 0.959)\) with the 14 conflicting assignments \((q = 0.770, P < 0.0007, t\)-test). Also, four of these individuals were identified by GENECLASS 1.0.02 (Cornuet et al. 1999) as samples that could not be grouped into any source population, and thus are likely to be migrants from an unsampled source population. A geographical evaluation of the 14 conflicting assignments revealed that 11 samples were in the region between genetic clusters 1 and 2 (Fig. 1a). The three subpopulations defined by STRUCTURE were used for genetic assignment tests requiring predefined subpopulations. The subpopulations in the later analyses and discussion are as follows: Rocky Mountains Front (RMF; \(n = 45\)), Crazybelts \((n = 19)\) and Gallatin \((n = 26)\) (Fig. 1a). If a sample was defined genetically in a population different to its geographical congeners, it was placed within its sampling group, not the assigned group. This correction ensures that population allele frequencies are not biased by the removal of possible immigrants.

Identification of migrants

The putative migrants identified by the assignment-based approaches are displayed in Table 2. STRUCTURE identified the smallest number of migrants \((n = 11)\). STRUCTURE was able to classify 73\% of the samples with a high degree of certainty \((q \geq 0.90)\). Only four of the samples \((3, 4, 6 \text{ and } 10)\) with probabilities lower than the threshold \((q < 0.90)\) were identified as migrants and placed in a geographical region different from sampling origin. Eleven samples \((13, 15, 16, 17, 18, 19, 20, 21, 22, 23 \text{ and } 24)\) were placed in the region of sampling origin but have \(q\)-values below the 0.90 threshold. These samples represent individuals with mixed or ambiguous ancestry, and each sample was identified as a migrant by one or more of the other assignment tests.

If the results of all four tests are combined, 25 individuals were classified as migrants or offspring of migrants. Only nine of the migrants \((1, 2, 3, 6, 7, 8, 9, 10 \text{ and } 11)\) were identified by all four methods. Of these individuals, eight were assigned to the same source population (or included the source population in the exclusion method) using all four methods. The exclusion method was not able to place two migrants into any of the sampled source populations \((2, 7)\) and placed six individuals into multiple source populations. Overall, 22 migrants were detected by the iterative method, and seven of these migrants were not identified by any other assignment method \((12, 19, 20, 22, 23, 24 \text{ and } 25)\). However, the results of the iterative method were consistent with the results of STRUCTURE in 97\% of the samples when applying the \(q \geq 0.90\) threshold. All the migrants detected by STRUCTURE were detected by the frequency test (Paetkau et al. 1995), and the frequency test classified five additional migrants \((13, 15, 16, 17 \text{ and } 25)\).

In the Bayesian exclusion test of GENECLASS, an individual is assigned to a population if its probability of originating in all other populations is less than a chosen threshold \((P < 0.10)\). The exclusion method was only able to assign 43\% of the samples with a probability of \(P < 0.10\). If a confidence threshold is not used, then individuals can be assigned to the population with the highest probability. In this data set, the average probability of assignment was \((P) = 0.54\). Fourteen migrants were identified using this method, and five could not be placed into any of the source populations \((2, 4, 7, 18 \text{ and } 21)\); Table 2), suggesting that the source population for these individuals was not sampled.

Direct vs. indirect methods

Pairwise \(F_{ST}\) values were calculated for the three sampling areas for the total data set, males only and females only (Table 3). The direct estimates of dispersal provided by the assignment tests are compared to indirect measures of dispersal (Table 4). The direct estimates suggested that the RMF and Crazybelts subpopulations exchanged very few migrant males/generation \((0–2.5)\) and no females while the RMF and Gallatin subpopulations exchanged more migrants/generation \((3–6.5)\); Table 4). The Crazybelts subpopulation also exchanged few migrants with the Gallatin subpopulation (Table 4). The indirect methods revealed a similar pattern of gene flow.

Levels of gene flow between males and females

The number of migrant males and females were calculated directly from each of the assignment tests (Table 4). For each subpopulation pair, the above results were compared to average assignment indices for males and females \((AI_f)\), average percentage of ancestry \((q)\) for males and females, \(F_{ST}\) estimates, and \(N_m\) estimates. The assignment index of individuals \((AI_f)\) was not statistically different between sexes \((P = 0.779);\) Table 5). In contrast, \(F_{ST}\) values were significantly different between males and females (Table 3).
Table 2 Migrant wolverines identified by four different assignment based approaches. Geographic assignment is shown for each method with corresponding statistical support. Individuals marked by * were identified as a migrant by all tests.

<table>
<thead>
<tr>
<th>Wolverine</th>
<th>Sex</th>
<th>Geographic origin</th>
<th>Bayesian structure</th>
<th>q</th>
<th>Iterative LOD</th>
<th>Geneclass frequency LOD</th>
<th>Geneclass Bayesian exclusion†</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Female Crazybelts</td>
<td>Gallatin</td>
<td>0.950</td>
<td>Gallatin</td>
<td>2.517</td>
<td>Gallatin</td>
<td>1.852</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>2*</td>
<td>Female Gallatin</td>
<td>RMF</td>
<td>0.968</td>
<td>RMF</td>
<td>4.775</td>
<td>RMF</td>
<td>3.364</td>
<td>none</td>
</tr>
<tr>
<td>3*</td>
<td>Female Gallatin</td>
<td>RMF</td>
<td>0.836</td>
<td>RMF</td>
<td>0.491</td>
<td>RMF</td>
<td>1.379</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>4</td>
<td>Female Gallatin</td>
<td>Crazybelts</td>
<td>0.835</td>
<td>Gallatin</td>
<td>0.623</td>
<td>Crazybelts</td>
<td>0.633</td>
<td>none</td>
</tr>
<tr>
<td>5</td>
<td>Female RMF</td>
<td>Gallatin</td>
<td>0.957</td>
<td>Gallatin</td>
<td>2.647</td>
<td>Gallatin</td>
<td>1.532</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>6*</td>
<td>Male Gallatin</td>
<td>Crazybelts</td>
<td>0.708</td>
<td>Crazybelts</td>
<td>0.438</td>
<td>Crazybelts</td>
<td>1.258</td>
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</tr>
<tr>
<td>7*</td>
<td>Male RMF</td>
<td>Gallatin</td>
<td>0.921</td>
<td>Crazybelts</td>
<td>4.127</td>
<td>Gallatin</td>
<td>3.049</td>
<td>none</td>
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<tr>
<td>8</td>
<td>Male RMF</td>
<td>Gallatin</td>
<td>0.983</td>
<td>Gallatin</td>
<td>2.989</td>
<td>Gallatin</td>
<td>3.153</td>
<td>RMF, Gallatin, Crazybelts</td>
</tr>
<tr>
<td>9</td>
<td>Male Crazybelts</td>
<td>Gallatin</td>
<td>0.942</td>
<td>Gallatin</td>
<td>1.692</td>
<td>Gallatin</td>
<td>1.865</td>
<td>RMF, Gallatin, Crazybelts</td>
</tr>
<tr>
<td>10*</td>
<td>Male RMF</td>
<td>Gallatin</td>
<td>0.771</td>
<td>Gallatin</td>
<td>3.880</td>
<td>Gallatin</td>
<td>2.459</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>11*</td>
<td>Male Crazybelts</td>
<td>Gallatin</td>
<td>0.913</td>
<td>Gallatin</td>
<td>2.078</td>
<td>Gallatin</td>
<td>1.251</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>12</td>
<td>Female RMF</td>
<td>RMF</td>
<td>0.924</td>
<td>Gallatin</td>
<td>0.331</td>
<td>RMF</td>
<td>3.161</td>
<td>RMF</td>
</tr>
<tr>
<td>13</td>
<td>Male Gallatin</td>
<td>Gallatin</td>
<td>0.514</td>
<td>Gallatin</td>
<td>0.944</td>
<td>RMF</td>
<td>0.126</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>14</td>
<td>Male Gallatin</td>
<td>Gallatin</td>
<td>0.965</td>
<td>Gallatin</td>
<td>6.691</td>
<td>RMF</td>
<td>4.516</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>15</td>
<td>Female Gallatin</td>
<td>Gallatin</td>
<td>0.844</td>
<td>RMF</td>
<td>1.890</td>
<td>Crazybelts</td>
<td>1.482</td>
<td>RMF, Gallatin, Crazybelts</td>
</tr>
<tr>
<td>16</td>
<td>Male RMF</td>
<td>RMF (Crazybelts)</td>
<td>0.856</td>
<td>Crazybelts</td>
<td>0.001</td>
<td>Crazybelts</td>
<td>1.090</td>
<td>RMF, Crazybelts</td>
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<tr>
<td>17</td>
<td>Male RMF</td>
<td>RMF</td>
<td>0.696</td>
<td>Crazybelts</td>
<td>1.078</td>
<td>Gallatin</td>
<td>0.358</td>
<td>RMF</td>
</tr>
<tr>
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<td>Crazybelts</td>
<td>0.537</td>
<td>RMF</td>
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<td>Crazybelts</td>
<td>0.225</td>
<td>none</td>
</tr>
<tr>
<td>19</td>
<td>Male RMF</td>
<td>RMF</td>
<td>0.729</td>
<td>Gallatin</td>
<td>1.426</td>
<td>RMF</td>
<td>0.592</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>20</td>
<td>Male RMF</td>
<td>RMF</td>
<td>0.598</td>
<td>Gallatin</td>
<td>2.792</td>
<td>RMF</td>
<td>0.076</td>
<td>RMF</td>
</tr>
<tr>
<td>21</td>
<td>Male Crazybelts</td>
<td>Crazybelts</td>
<td>0.639</td>
<td>RMF</td>
<td>1.328</td>
<td>Crazybelts</td>
<td>1.409</td>
<td>none</td>
</tr>
<tr>
<td>22</td>
<td>Male RMF</td>
<td>RMF</td>
<td>0.802</td>
<td>Gallatin</td>
<td>1.958</td>
<td>RMF</td>
<td>1.742</td>
<td>RMF</td>
</tr>
<tr>
<td>23</td>
<td>Male RMF</td>
<td>RMF</td>
<td>0.747</td>
<td>Gallatin</td>
<td>0.938</td>
<td>RMF</td>
<td>0.639</td>
<td>RMF, Gallatin</td>
</tr>
<tr>
<td>24</td>
<td>Male RMF</td>
<td>RMF</td>
<td>0.829</td>
<td>Gallatin</td>
<td>0.180</td>
<td>RMF</td>
<td>1.152</td>
<td>RMF</td>
</tr>
<tr>
<td>25</td>
<td>Female RMF</td>
<td>RMF</td>
<td>0.909</td>
<td>Gallatin</td>
<td>0.047</td>
<td>RMF</td>
<td>2.228</td>
<td>RMF</td>
</tr>
</tbody>
</table>

Total number of migrants 11 22 16 14

†For the exclusion method, a threshold of 0.10 was used however the subpopulations in bold indicate the most likely subpopulation; LOD = likelihood ratio, RMF = Rocky Mountain Front.
randomization approach of \textit{fstat} revealed that the average $F_{ST}$ estimate across the subpopulations for males ($\theta = 0.075$) was significantly lower than females ($\theta = 0.139; P = 0.004$). The estimated percentage of ancestry ($q$) for males ($q = 0.875$) was significantly lower than for females ($q = 0.934$) in all of the subpopulations ($P = 0.007$, Kruskal–Wallis sign test).

**Discussion**

**Detection of subpopulations in Montana**

The use of highly polymorphic microsatellite loci and assignment tests has greatly increased our potential for understanding population structure across a landscape. The Bayesian approach of \textit{structure} detected significant genetic structure for Montana wolverines without using prior geographical information and has proved to be a powerful method in many instances where genetic differentiation is high (Pritchard \textit{et al}. 2000; Randi \textit{et al}. 2001; Manel \textit{et al}. 2002; Mauget \textit{et al}. 2002; Randi & Lucchini 2002). The appropriate choice of the number of subpopulations, $K$, was not straightforward for this data set as optimal likelihood modes were observed at $K = 3$ and $K = 5$. Based on higher $q$-values, greater geographical concordance, and recommendations from the literature to choose the smallest value of $K$ that captures the major structure in the data set when differences between likelihood values are small (Pritchard \textit{et al}. 2000; Pritchard & Wen 2002), we believe $K = 3$ is the optimal choice for our data set. The frequency-based iterative approach was also used to evaluate population structure and support for the $K = 3$ grouping. A high degree (84%) of concordance in population grouping was observed between methods, and individual assignments of the iterative method agreed with the results of \textit{structure} in 97% of the samples when using a threshold value of $q \geq 0.90$.

Our analyses indicate clearly that Montana wolverines are not a panmictic population and at least three wolverine subpopulations exist within the state. This result is surprising, given the geographical proximity of the subpopulations and the dispersal capabilities of wolverines. These subpopulations are separated by approximately 300 km, a distance within the range of wolverine annual movements (Gardner 1985; Magoun 1985). The detection of significant genetic structure across this spatial scale contrasts with results observed in wolverine populations of Canada and Alaska (Kyle & Strobeck 2001, 2002). Kyle & Strobeck (2001, 2002) reported little genetic structure ($F_{ST} = 0.001–0.03$) across distances of 1000–2000 km within a continuous distribution of wolverines in northern Canada and Alaska. The higher genetic structure ($F_{ST} = 0.08–0.10$) observed among Montana wolverines suggests that habitat in Montana is much more fragmented than habitats in Canada and Alaska. Kyle & Strobeck (2002) also report increasing $F_{ST}$ values and genetic structure when analysing wolverine populations from more human fragmented landscapes in southern Canada and Idaho.

Human disturbance of the landscape may be responsible for this difference. Topographic features such as lakes and mountain ranges do not appear to limit wolverine movement (Kyle & Strobeck 2001, 2002); however, wolverines do not tolerate land-use activities that alter habitats permanently, such as agriculture, mining activities and human settlement (Banci 1994; Carroll \textit{et al}. 2001). Predictive habitat modelling has revealed that wolverines use a variety of vegetation types in the Rocky Mountains, and that low

<table>
<thead>
<tr>
<th>Method</th>
<th>Gallatin ↔ CB</th>
<th>CB ↔ RMF*</th>
<th>RMF* ↔ Gallatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bayesian \textit{structure}</td>
<td>2.5 (4:1)</td>
<td>0.0 (0:0)</td>
<td>3.0 (3:3)</td>
</tr>
<tr>
<td>Iterative</td>
<td>2.0 (3:1)</td>
<td>2.5 (5:0)</td>
<td>6.5 (7:6)</td>
</tr>
<tr>
<td>\textit{geneclass} frequency</td>
<td>3.0 (3:3)</td>
<td>0.5 (1:0)</td>
<td>4.5 (6:3)</td>
</tr>
<tr>
<td>\textit{geneclass} Bayesian exclusion</td>
<td>0.0 (0:0)</td>
<td>2.0 (3:1)</td>
<td>2.5 (4:1)</td>
</tr>
<tr>
<td>Indirect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slatkin $N_m$</td>
<td>1.16</td>
<td>0.67</td>
<td>3.59</td>
</tr>
<tr>
<td>Wright $N_m$</td>
<td>2.55</td>
<td>2.20</td>
<td>2.76</td>
</tr>
</tbody>
</table>

*RMF = Rocky Mountain Front, CB = Crazybelts.

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population density and low road density were the most useful predictors for modelling observations of wolverines in Northwest USA (Carroll et al. 2001; Rowland et al. 2003). Habitats near roads and logged areas and open areas with limited cover were also shown to be selected against (Hornacker & Hash 1987; Krebs & Parfitt 2002). Based upon these studies, the most likely attributes influencing the observed population genetic structure in Montana are major roads, cities and agricultural areas in the lowland valleys. Interstate highways 15 and 90 are two major roadways that bisect the study area along with human populations inhabiting the cities and surrounding areas of Butte, Bozeman and Missoula, Montana (Fig. 2). Even recreational snowmobile use may affect wolverine distribution (Hornacker & Hash 1987; Krebs & Parfitt 2002). Our findings highlight the sensitivity of wolverines to landscape changes involving human occupation and land-use and the importance of identifying large tracts of undisturbed habitat to protect these species.

Population genetic studies of other large carnivores in Montana have documented fragmentation and potential loss of critical habitat. Microsatellite analysis of the Northern Continental Divide brown bear (Ursus arctos) population in the northwestern corner of Montana and the Yellowstone population in the southwestern corner also revealed moderate $F_{ST}$ values (0.12), and lower levels of diversity in the Yellowstone population (Paetkau et al. 1998; Waits et al. 1998; Miller & Waits 2003). Ostheimer (1998) evaluated gene flow and genetic diversity among black bear (U. americanus) populations in Montana using microsatellites. Results of his study indicated higher gene flow and diversity among bears in the northern portion of Montana compared to bears in the southern and eastern portions. He also concluded that populations in different mountain ranges were becoming isolated.

**Inferring levels of gene flow**

Because it is difficult to quantify gene flow (Slatkin 1987), the results of multiple methods with different underlying models can be used to provide minimum and maximum estimates of gene flow.

The concordance among tests can be evaluated to verify the accuracy of results for empirical studies where the assumptions for each test may not always be met. Furthermore, the identification of the source population of a sample may require more than one assignment test for verification (Manel et al. 2002). In our case, the ‘true’ source population for each individual was not known, so multiple methods were used to verify placement and provide a range of gene flow estimates to assist managers. We caution that our estimates of gene flow are presented as relative measures of connectivity between populations and will not represent true numbers of migrants per generation.

Cornuet et al. (1999) revealed through simulations that 100% accuracy of population assignment can be obtained under the following conditions: $F_{ST}$ estimates of 0.10, 30–50 samples per population and 10 loci with an average heterozygosity, $H_E = 60\%$. High accuracy for STRUCTURE can be obtained under similar conditions (Pritchard et al. 2000; Rosenberg et al. 2001; Manel et al. 2002). Despite meeting most of these recommended conditions in our data set, some discordance was evident among the different assignment test approaches for inferring putative migrants. When including results of all methods 25 individuals were classified as migrants, but only nine of these individuals were identified as migrants by all four methods. The number of migrants identified by a single method ranged from 11 to 22. In our analyses, the Bayesian approach of STRUCTURE (Pritchard et al. 2000) was more conservative and identified the lowest number of migrants. The frequency-based
iterative method (Vasquez-Dominguez et al. 2001) suggested a greater degree of gene flow between subpopulations than all other methods and identified seven migrants that were not detected by any other method. The lack of agreement among methods suggests caution at basing conclusions and management recommendations on a single assignment test approach.

Higher concordance was observed among assignments from STRUCTURE (Pritchard et al. 2000) and the GENECLASS frequency approach (Cornuet et al. 1999) than when comparing either method to the Bayesian exclusion approach of GENECLASS (Cornuet et al. 1999). STRUCTURE simultaneously tests the probability of assignment for one or more populations, which may lead to higher accuracy (Maudet et al. 2002). The approach of the exclusion method considers each population separately and assigns an individual to a population if and only if its probability of originating in all populations is less than the threshold (Cornuet et al. 1999). In this study, the exclusion method was not able to classify an individual to a sole source population despite sufficient genetic differentiation ($F_{ST} = 0.08 – 0.10$) and moderate genetic variation ($H_E = 0.50$; $A = 4.5$). This lack of power has been documented in a number of empirical and simulated data sets (Eldridge et al. 2001; Manel et al. 2002; Maudet et al. 2002). However, the exclusion method is valuable because it is the only method that provides the option of rejecting placement in all sampled populations, and studies may not always sample the true population of origin for highly mobile species such as wolverines. In our data set, the exclusion method suggests that five wolverines may be migrants from unsampled populations.

Sex-biased dispersal

Prior to the development of assignment tests, sex biases in dispersal were evaluated by comparing differentiation at both nuclear and mitochondrial DNA (Paetkau et al. 1995; Fitzsimmons et al. 1997), pairwise $F_{ST}$ and allelic divergence (Kawata 1985; Rassman et al. 1997; Balloux et al. 1998) and relatedness values (Ishibashi et al. 1997; Knight et al. 1999; Surridge et al. 1999). Rassman et al. (1997) showed that significantly lower $F_{ST}$ estimates for males relative to females could signal sex-biases in dispersal. The percentage of ancestry ($q$) estimated by STRUCTURE has not been used previously to measure differences in gene flow between males and females, but may provide relevant information. A low value of $q$ indicates a low probability that the individual’s genome originated in the respective subpopulation due to admixture and migration among subpopulations. In contrast, a significantly higher value of $q$ may be more likely in the philopatric sex. Our results show that males had significantly lower values of $q$ in each of the subpopulations than females, indicating a male bias in dispersal. $F_{ST}$ results further supported this result as $F_{ST}$ estimates were significantly higher for females than males. Previous genetic studies also support a male bias in dispersal for wolverines. Wilson et al. (2000) analysed allozyme and mitochondrial (mtDNA) from wolverines in the Northwest Territories of Canada and detected significant genetic structure for mtDNA, but not allozymes, suggesting female philopatry. Our results support the hypothesis that males are the predominant dispersers, and females disperse at lower frequencies (Banci 1994).

The assignment indices ($A_{1c}$) for male and female wolverines were not statistically different, suggesting that dispersal was equivalent for both sexes. There are a number of possible explanations for this inconsistency compared to $F_{ST}$ and $q$-values. First, the $A_{1c}$ method may be less powerful, as suggested by simulations (Goudet et al. 2002). $F_{ST}$ estimates outperformed all other methods in cases where the entire population could not be sampled and the sex bias was less extreme (Goudet et al. 2002), as might be the case for Montana wolverines. Secondly, male and female migration may be equal but females who migrate may be less likely to rear offspring successfully. Direct methods such as $A_{1c}$ can detect if dispersal has taken place, but cannot reveal if genes were successfully passed on to the next generation (Slatkin 1987). Indirect estimates, which use allele frequency data such as $F_{ST}$, can reveal differences in reproductive success over time (Slatkin 1987). A similar rate of male and female dispersal could be inferred by the $A_{1c}$ test, yet the cost of dispersal may outweigh the benefits (measured by reproductive success) in females more than males in a polygamous mating system. Males have been documented to disperse as yearlings or subadults (Gardner 1985; Magoun 1985; Banci 1987) and gain more breeding opportunities by dispersing (Vangen et al. 2001). Occasional reports of female dispersal have been documented, but females generally remain philopatric to their natal areas, due presumably to benefits of known food resources and denning sites (Vangen et al. 2001).

Conservation and management implications

While some discrepancy exists as to the source of some putative migrants, relative gene flow estimates between the subpopulations are consistent for all direct and indirect methods. Our estimates of gene flow are presented as relative measures of connectivity between populations and will not represent true numbers of migrants per generation. However, these estimates provide a useful index of gene flow to assist management. The Crazybelts subpopulation is relatively isolated (0–2.5 migrants/generation), with higher gene flow between the RMF and Gallatin subpopulations (3–6.5 migrants/generation). These levels of gene flow are low compared to studies of wolverines in Alaska and northern Canada (Kyle & Strobeck 2001, 2002) and this restricted movement warrants further research. These results

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have important implications for harvesting wolverines in Montana. The wolverine is especially susceptible to harvest in Montana due to reduced levels of gene flow, low reproductive rates and need for large areas of undisturbed habitat. A lack of movement among geographical regions where harvest is occurring may be detrimental for the re-colonization of vacant habitats. Vangen et al. (2001) published the first long-term study of wolverine dispersal in a harvested population in Scandinavia. That study documented the capability of wolverines to travel among subpopulations, yet found a large proportion of vacant female habitats. High turnover rates resulted in more sedentary females and lower rates of female dispersal (Vangen et al. 2001). If female dispersal is limited, the colonization of compromised populations will be slower (Vangen et al. 2001). Our study suggests that a similar pattern of dispersal may occur in Montana and warrants attention.

It has been proposed that one migrant per generation (OMPFG) is sufficient to prevent population differentiation due to genetic drift (Kimura & Ohta 1971; Franklin 1980; Frankel & Soule 1981; Allendorf 1983). However, recent studies have suggested that up to five to 10 migrants per generation may be more reasonable for natural populations (Frankel & Soule 1981; Mills & Allendorf 1996). The apparent isolation within the Crazylbelts subpopulation suggests that this region may not be re-colonized as easily as other regions in Montana. The RMF and Gallatin subpopulations are connected with a higher level of gene flow, suggesting that habitat corridors still exist between the subpopulations. Additional research is needed to evaluate the specific habitat needs of wolverines in order to re-establish and sustain connectivity among Montana subpopulations. Research is also needed to evaluate the connectivity of Montana subpopulations to other populations in the lower 48 states and southern Canada.

Conclusions

This study demonstrates the utility of assignment tests for delineating subpopulations and simultaneously estimating levels of gene flow. This approach has great potential for the identification of populations across a continuous landscape and may revolutionize population genetic analyses. The use of multiple assignment tests to detect potential migrants revealed a substantial degree of discrepancy, and researchers should avoid basing conclusions and management recommendations on a single assignment test approach. Additional computer simulations and evaluations of samples with known population origin are needed to evaluate the strengths and weaknesses of different assignment based approaches. The detection of reduced gene flow levels among wolverine populations in Montana adds to a growing body of literature that documents an increasingly fragmented landscape for large carnivores in the northwestern United States.

For long-term population persistence of these species, conservation biologists and wildlife managers must identify specific habitat requirements to delineate critical habitat and maintain connectivity of populations.

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